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Advanced Imaging Approaches to Characterize Stromal and Metabolic Changes in In Vivo Mammary Tumor Models

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14. ABSTRACT

The purpose of this study is to investigate the connection between cancer cell progression and key factors in the cellular microenvironment such as metabolism and changes in the ECM, specifically by examining the effects of collagen density on cellular metabolism in breast cancer cells. Normal (MCF10A) and invasive (MCF10-Ca1d) breast epithelia cells were cultured in 2D and, to be more relevant, in 3D by suspending the cells in high and low density collagen gels. To stress cellular metabolism, the cells were treated with either DFOM, to mimic a hypoxic response, or 2DG to induce a hypoglycemic environment. Fluorescence lifetime data were then collected for the NADH within the cells. In 2D, the MCF10A cells show the trend of having a shorter free fractional component than MCF10-Ca1d cells. In 3D, increasing collagen density caused an increase in the free fraction of NADH for nearly all cell type and treatment conditions. In most cases DFOM or 2DG caused a decrease in the free fraction of NADH. This in vitro data provides valuable FLIM characterization and toward future intravital studies. Preliminary fluorescence lifetime images were also collected intravitally through a mammary imaging window implanted in a female, PyVT positive, Col1a1 heterozygote, mouse.

15. SUBJECT TERMS
Multiphoton Fluorescence Excitation Microscopy, Fluorescence Lifetime Imaging Microscopy, Extracellular Matrix, Mammary Imaging Window

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Introduction:

Increased breast density is correlated to a 4-6 fold increased risk in developing breast cancer, yet the physical and molecular mechanisms are poorly understood (*1*). We have found that alignment changes in the collagen matrix that contribute to this density can be specifically correlated to poor clinical prognosis (*2*). These changes in collagen alignment that we have coined "Tumor Associated Collagen Signatures" (TACS) (*3*) can be uniquely probed with advanced imaging techniques such as Second Harmonic Generation (SHG) (*4*) and multiphoton laser scanning microscopy (MPLSM) (*5*) that can non-invasively examine collagen and cells respectively in in vitro and in vivo cancer models. Due to the importance of TACS in breast cancer, our lab has been increasingly focused on characterizing the relationship between the extracellular matrix (ECM) and breast cancer progression. My project focuses on examining the cellular microenvironment in breast cancer and investigating the connection between cancer cell progression and key factors in the microenvironment such as metabolism and changes in the ECM. Specifically my research aims to examine the possible effects of collagen density on cellular metabolism in breast cancer cells.

Cellular metabolism is known to be a key factor in cancer cell progression due to the Warburg Effect (*6*). The Warburg Effect is characterized by a switch from aerobic metabolism, which produces large amounts of energy in the form of the co-enzyme Adenosine triphosphate (ATP), to anaerobic metabolism, which supports cell growth and proliferation. To study metabolism, I have been using Fluorescence Lifetime Imaging Microscopy (FLIM) (*7*) to examine Nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide (FAD), intermediate metabolites with fluorescence properties. FLIM is a powerful technique for this as unlike invasive biochemical methods it can be used *in vivo* to detect changes in NADH binding and other local changes due to FLIM being sensitive to microenvironment chemical changes. Our lab has shown previously that there is a shift in fluorescence lifetime of NADH and FAD in histology slides of developing breast tumors compared to healthy breast epithelia (*8*).

We have preliminary data that suggest that the cellular processes that we observe with TACS are associated with metabolic changes. We proposed a series of advanced imaging experiments to examine this metabolism and collagen interplay at the cellular level. To understand this shift and its cause, I am using MCF10a and MCF10-Ca1d cells in culture to characterize changes in the fluorescence lifetime of NADH. I intend to use this characterization to understand shifts in fluorescence lifetime collected by intravital imaging using a mammary imaging window implanted in a mouse over a developing mammary tumor. Year 1 of this fellowship has focused on this characterization in two-dimensional and three-dimensional cell culture as well as mastery of the mammary imaging window technique and associated imaging techniques, which has resulted in a methods manuscript (*9*).

Body:

My statement of work focused on two main tasks, the first to be completed in year 1, and the second to begin in year 2.

Task 1: Determine the relationship between collagen density and cellular metabolism in vitro. (months 1-8)

There are two main goals for this task which I have completed in year 1 of my fellowship. The first goal is to characterize endogenous optical biomarkers as a measure of cellular metabolism in vitro under known hypoxic and hypoglycemic stressors. MCF10A (normal breast epithelial) and MCF10-Ca1d cells (invasive breast cancer cells lines) were cultured in two dimensions, using typical cell culture protocol, as well as in three dimensions, suspending the cells in gels made of type 1 collagen in high and low density. The three dimensional studies are important as they are more physiologically relevant than cells grown in 2D culture formats.

The cells are then stressed in order to encourage a metabolic shift from aerobic metabolism to anaerobic metabolism, or glycolysis. Hypoxia occurs when cells are in a low oxygen environment, causing activation of hypoxia inducible factors, which lead to transcription of hypoxia response elements and a switch to glycolysis. Hypoglycemia occurs when cells are deprived of glucose, limiting glycolysis and therefore also limiting oxidative phosphorylation. The cells were treated for 24 hours with either the iron chelator, Deferoxamine (DFOM), to mimic a hypoxic response, or the glucose molecule 2-Deoxy-D-glucose (2DG) to induce a hypoglycemic environment. Fluorescence lifetime data were then collected for the NADH within the cells (Figure 2).

In two dimensions, the MCF10A cells have a longer fluorescence lifetime (as defined by ($Tau\ Mean = \frac{A1*Tau1+A2*Tau2}{100\%}$)) and shorter free fractional component than MCF10-Ca1d cells, though not statistically significantly different (Mixed Procedure Test- see below). As a current and future step, I am focusing on the FLIM analysis as this is a major component of accurate and reproducible FLIM data particularly data that is collected in heterogeneous in vivo environments such as what I propose. There are many parameters to consider when identify what changes in lifetime are not only real but statistically significant. We have been working closely with experts in the field including the developers of the software we use predominantly for this, Becker and Hickl's SPCImage. Software parameters such as signal thresholding and binning can improved the statistics when adjusted correctly. This is due to not only the biological environment being variable and complex but also experimental conditions such as media components that can also contribute to signal background and thus would need correction. My preliminary experiments with this have suggested that by increasing the threshold and binning more reliable fits can be obtained.

When cells are cultured on two-dimensional surfaces, neither DFOM nor 2DG statistically affect MCF10A or MCF10-Ca1d cells (Figure 3 and 4). However, the trend appears that 2DG treatment reduced the free fraction of NADH to the same level as the MCF10a cells (Figure 4B).

The second goal of this task is to measure changes in cellular metabolism in response to changes in collagen density in vitro. Increasing collagen density caused a decrease in fluorescence lifetime and an increase in the free fraction of NADH for nearly all cell type and treatment conditions (Figure 5 and 6). The effect of DFOM in 3D culture was more subtle. In most cases DFOM treatment or 2DG treatment caused a decrease in the free fraction of NADH, but for the MCF10-Ca1d cells in high density, DFOM treatment caused an increase in the free fraction of NADH (Figure 5B) and for the MCF10A cells in high density, 2DG caused an increase in the free fraction of NADH (Figure 6B).

Task 2: Measure cellular metabolism associated with different collagen densities intravitally. (months 9-36)

My goal for year 1 was to optimize measurement of cellular metabolism in the mouse model which I have achieved (*10*). Preliminary fluorescence lifetime images have been collected intravitally through a mammary imaging window implanted in a female, PyVT positive, Col1a1 heterozygote, mouse (Figure 7). A paper has been written for Microscopy and Microanalysis on this technique.(*9*)

Statistical Analysis:

Data were analyzed using SPCImage to fit the measured fluorescence lifetime decay curves to a bi-exponential model generating a long and short lifetime component as well as the fractional contribution of each. These numbers were generated for each pixel in the image above the background threshold.

All data points from images collected were averaged for the same experimental conditions on the same day. Statistical significance was tested using the statistical software package SAS. An unequal variance model was implemented with the procedure MIXED. The model included the fixed effects - treatment, cell type and density and all possible interactions as well as the random effect - day. This unequal variance (by day) model was chosen based on results of various likelihood ratio tests. There was statistically significant day to day variation possibly caused by laser fluctuation or cell density prior to plating. Significance is determined by $\alpha<0.05$.

Troubleshooting:

Acquisition: During the course of these experiments, excellent progress was made in software development for FLIM acquisition and analysis, for which I was a predominant beta tester. Formerly, FLIM data were collected using two programs, WiscScan, an in-house software to control the microscope, and SPCM, a commercial software from Becker & Hickl to control FLIM data acquisition. This protocol was cumbersome and extremely slow. Imaging 8 samples (a typical 3D experiment) would take 12 hours of active imaging at the microscope. Because we were able to add FLIM functionality to WiscScan, we were able to utilize features already available in WiscScan including z-stack, time series, and montage collection. So instead personally setting up each 120 second image collection, we are now able to select multiple fields of view, and software will collect and save z-stacks of lifetime images at each field of view. With these improvements, I have obtained a 33% decrease in the time it takes to complete the experiments. This is key, as these experiments can take 10-12 hours.

FLIM Analysis: Additionally, we have developed SLIM Plugin, a FLIM analysis plugin for ImageJ, that is an open-source alternative to the commercial software available from Becker & Hickl. Although Becker & Hickl have added such features as batch processing (which is crucial for the large amounts of data I am collecting), there is still a lot of data massaging that the commercial software developers are not keen to reveal. By having an open source alternative, data analysis is extremely transparent. Additionally, because SLIMPlugin is a part of ImageJ, other features in ImageJ can be used in conjunction with SLIM Plugin. This is especially

important for data representation in publications. The most common way data that was analyzed in SPCImage is presented in publication is using a screenshot of the program because the images cannot be exported.

I will be presenting SLIM Plugin which has been developed by our group as an open source plugin for ImageJ for fluorescence lifetime analysis at Microscopy and Microanalysis 2013 in Indianapolis in August.

Future Directions:

Our lab has complimentary data using a luciferase assay to demonstrate that this concentration of DFOM does activate HIF-1 alpha. We will be writing up the in vitro DFOM data this year and intend to use a lactate assay to form a biochemical story around these data (*11*).

I will also continue, as stated in my original statement of work, to measure fluorescence lifetime in a mouse model using xenografts in high and low density collagen. MCF10CA-1D cells will be cultured in low and high-density collagen gels, then implanted the mammary gland of athymic nude mice. We will then implant an optical imaging window into the skin of these mice above the tumor. We will then collect SHG from collagen and FLIM data for NADH using an MPM system specifically designed for intravital imaging. Fluorescence lifetime will be calculated using SPCImage software (Becker and Hickl), TRI2 software (Paul Barber, Oxford and LOCI), and SLIM Plugin (LOCI). TACS will be analyzed using the Curvelet signal processing software, written at LOCI. We expect to find TACS-3 on the border between the xenograft and native tissue. We expect to measure an increase in fluorescence intensity and lifetime in tumor cells located in areas with TACS-3.

Key Research Accomplishments:

- Characterized endogenous optical biomarkers as a measure of cellular metabolism in vitro under known hypoxic and hypoglycemic stressors.
- Measured changes in cellular metabolism in response to changes in collagen density in vitro.
- Optimized measurement of cellular metabolism in mouse model.

Reportable Outcomes:

PA Young, A Grislis, PR Barber, PJ Keely, KW Eliceiri. Data Processing for Time-Domain Fluorescence Lifetime Imaging Microscopy, in Proceedings of Microscopy and Microanalysis 2013, Indianapolis, IN, USA, August 4-8, 2013. (in preparation)

D Inman, PA Young, J Szulczewski, PJ Keely, KW Eliceiri. Novel Intravital Imaging Approaches to Characterize Collagen Alignment in Defined Mammary Tumor Models. Microscopy and Microanalysis 2013. (in preparation)

Conclusion:

This first year of research has provided valuable characterization data to be applied to intravital studies in year 2 and 3. The *in vitro* data I collected served two important roles in preparing for my *in vivo* characterization. As discussed above there were a number of acquisition and analysis methods that needed to be refined which are best done in vitro. As well the data collected so far helped inform the experimental design for the intravital work. In addition to the Mammary Imaging Window paper, we are preparing another article on our in vitro discoveries that will include my FLIM data as well as the upcoming lactate assays.

References:

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11. J. M. Squirrell *et al.*, *PLoS One* **7**, e43708 (2012).

Supporting Data:

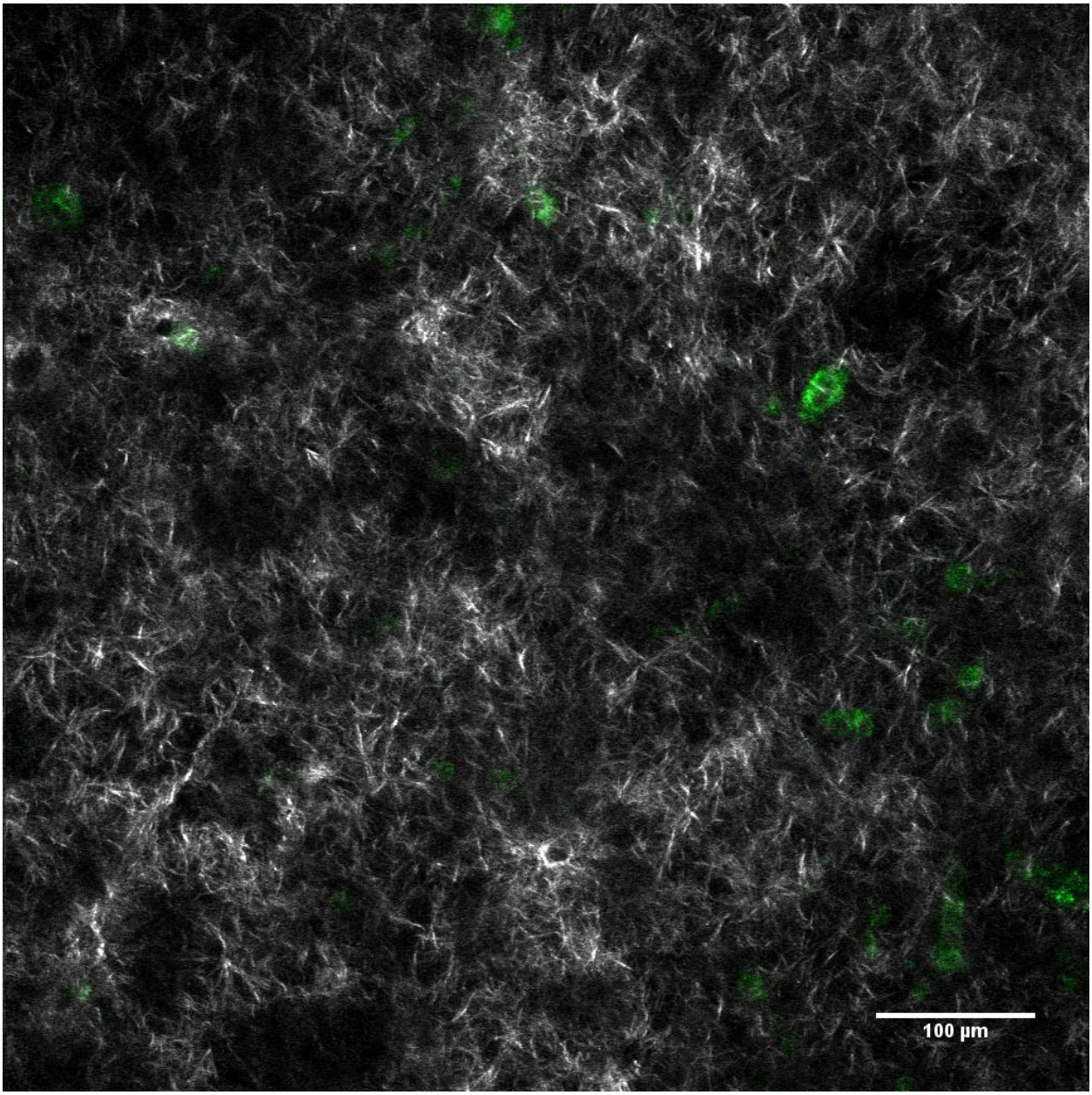


Figure 1: Metabolism in cancer cells imaged in 3D Collagen Model. MCF10-Ca1d cells 60 microns deep in 3mg/mL type 1 collagen (low density). NADH was excited using 780nm and collected through a 457/50 bandpass filter (green). SHG from the collagen was generated using 890nm and collected through a 445/20 bandpass filter (gray). A Nikon 20X Air Immersion lens (NA 0.75) was used for collection.

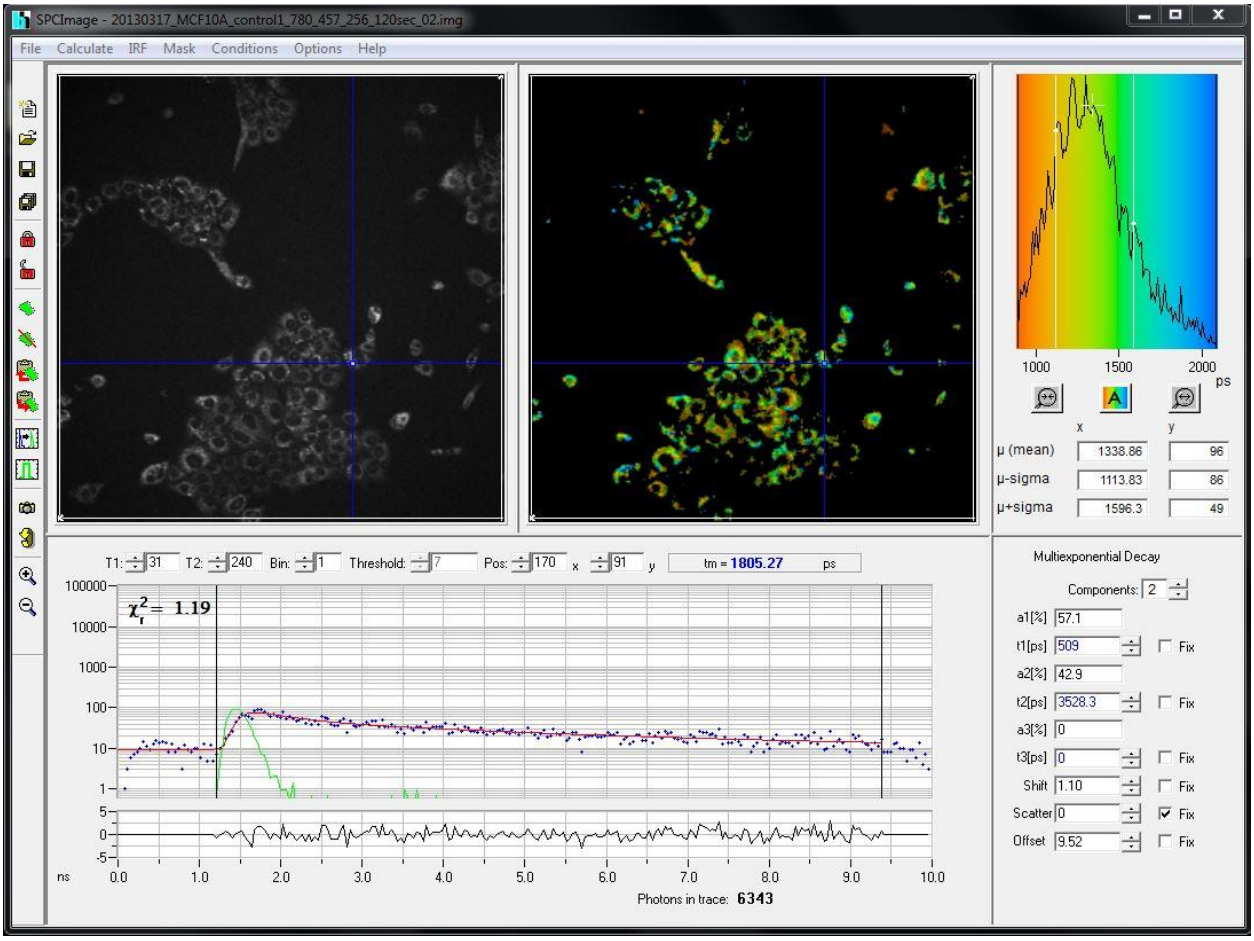


Figure 2: FLIM Measurement of NADH. Fluorescence lifetime image of NADH in untreated MCF10A cells collected with MPSLM at a wavelength of 780nm with a Nikon 20X Air Immersion lens (NA 0.75).

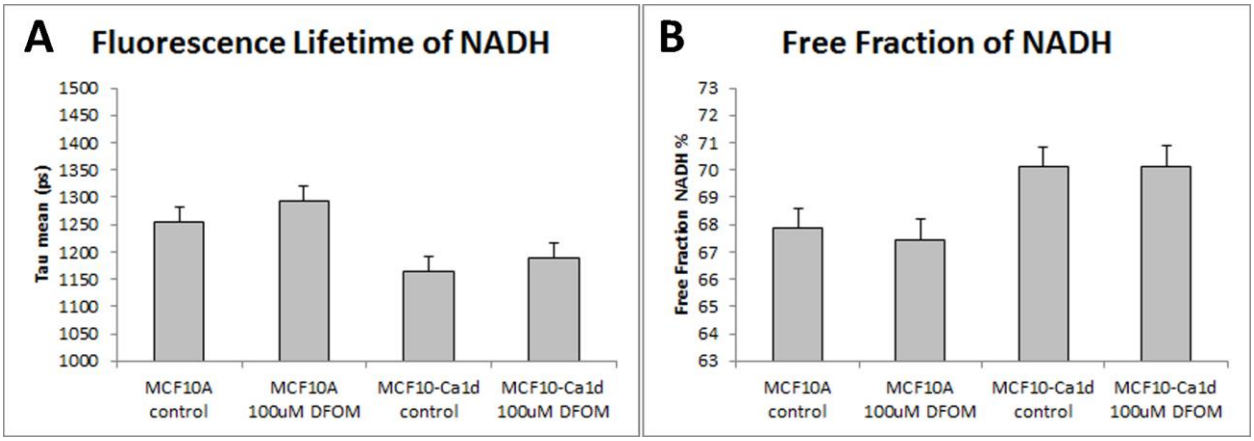


Figure 3: Fluorescence lifetime (A) and free fractional component (B) of NADH in MCF10A vs. MCF10-Ca1d with 100uM DFOM. There was not a significant difference ($\alpha<0.05$) between control and treated cells however tau mean and the free fraction of MCF10A cells treated with DFOM were significantly different from MCF10-CA1D treated and control cells indicating the trend. Fluorescence lifetime fitting will be repeated with increased threshold and binning and is expected to improve the statistics.

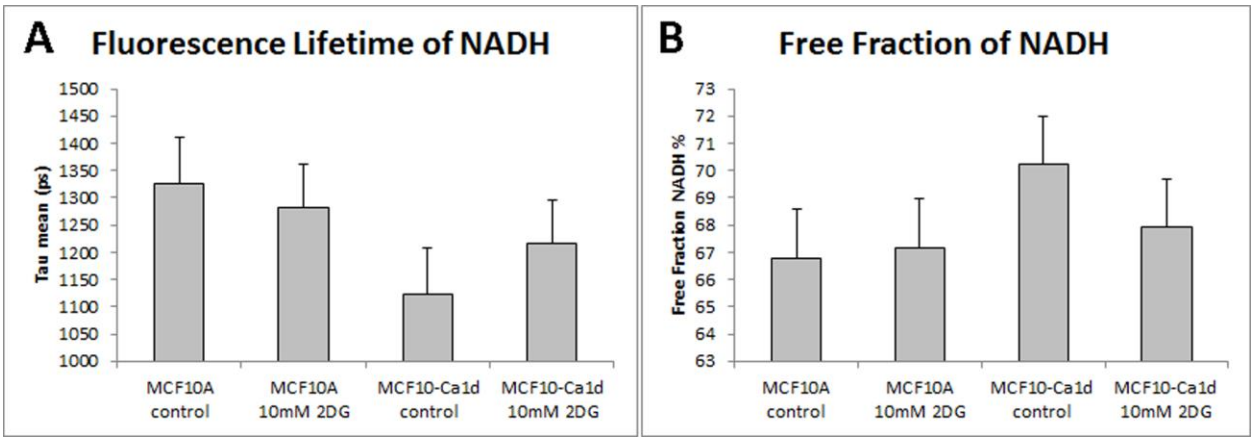
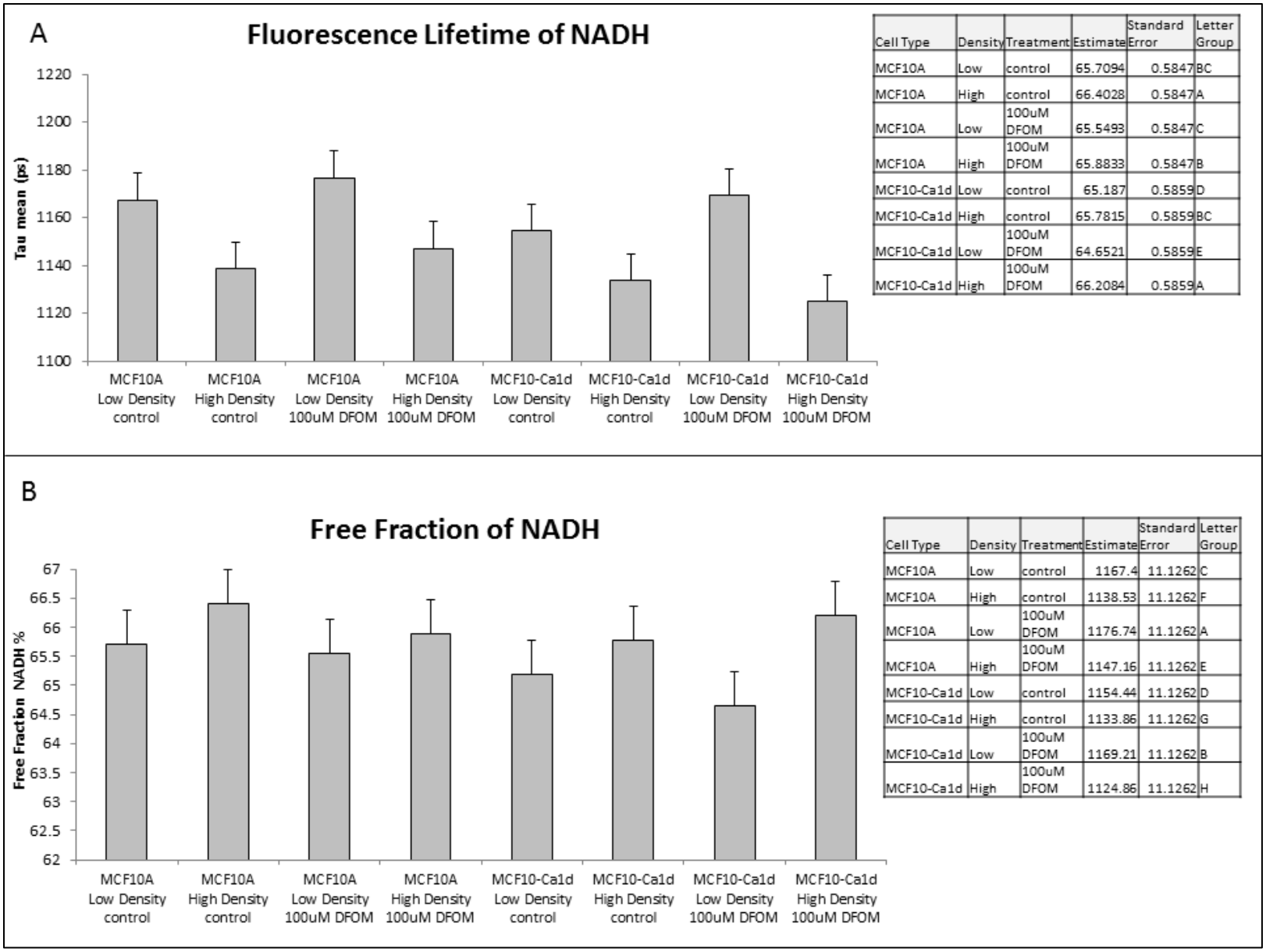


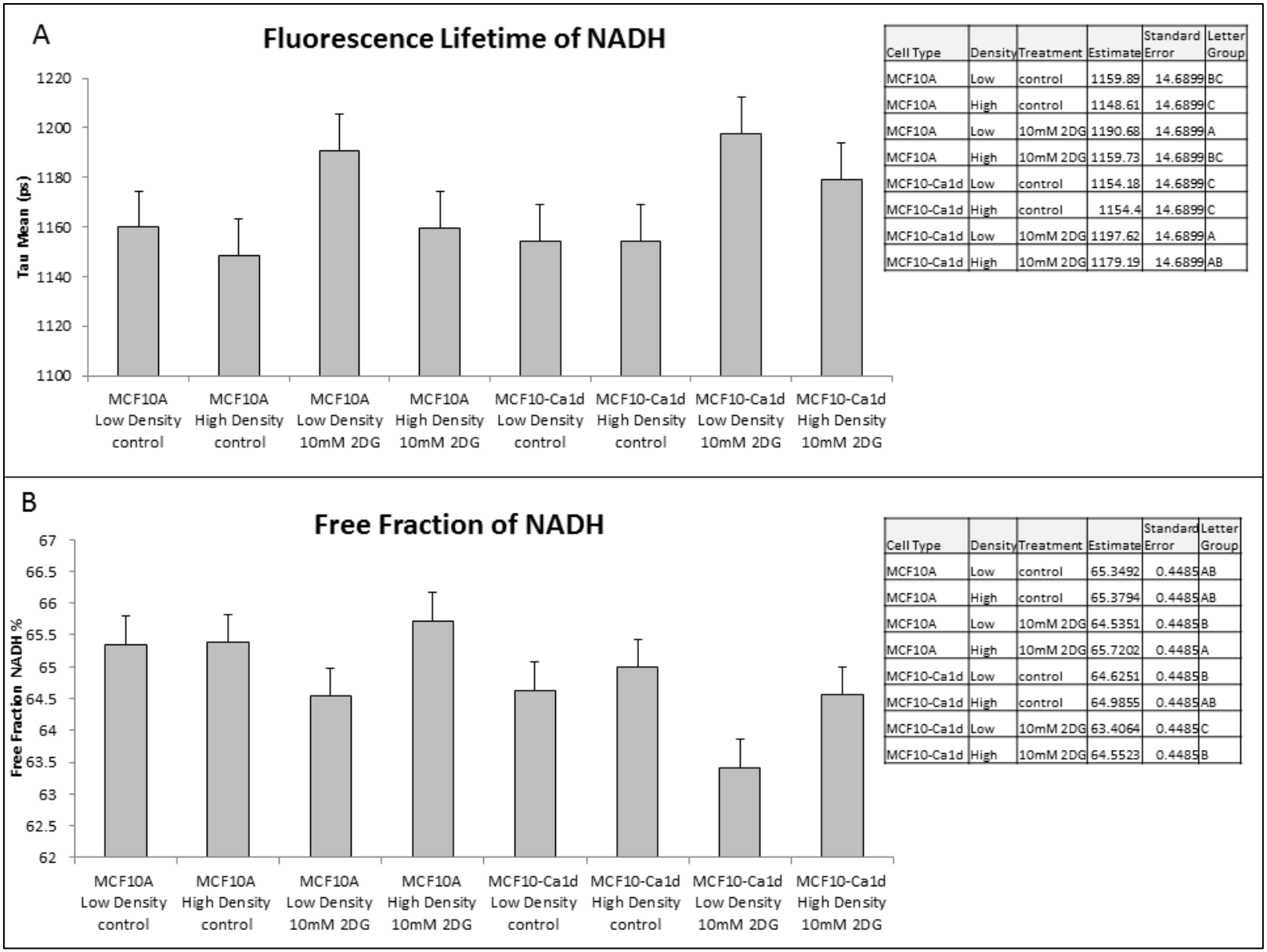
Figure 4: Fluorescence lifetime (A) and free fractional component (B) of NADH in MCF10A vs. MCF10-Ca1d with 10mM 2DG. There was not a significant difference ($\alpha<0.05$) between control and treated cells however the trend implies a reduction in free fraction of NADH in MCF10-Ca1d cells back to the levels in MCF10A cells. Fluorescence lifetime fitting will be repeated with increased threshold and binning and is expected to improve the statistics.



Cell Type	Density	Treatment	Estimate	Standard Error	Letter Group
MCF10A	Low	control	65.7094	0.5847	BC
MCF10A	High	control	66.4028	0.5847	A
MCF10A	Low	100uM DFOM	65.5493	0.5847	C
MCF10A	High	100uM DFOM	65.8833	0.5847	B
MCF10-Ca1d	Low	control	65.187	0.5859	D
MCF10-Ca1d	High	control	65.7815	0.5859	BC
MCF10-Ca1d	Low	100uM DFOM	64.6521	0.5859	E
MCF10-Ca1d	High	100uM DFOM	66.2084	0.5859	A

Cell Type	Density	Treatment	Estimate	Standard Error	Letter Group
MCF10A	Low	control	1167.4	11.1262	C
MCF10A	High	control	1138.53	11.1262	F
MCF10A	Low	100uM DFOM	1176.74	11.1262	A
MCF10A	High	100uM DFOM	1147.16	11.1262	E
MCF10-Ca1d	Low	control	1154.44	11.1262	D
MCF10-Ca1d	High	control	1133.86	11.1262	G
MCF10-Ca1d	Low	100uM DFOM	1169.21	11.1262	B
MCF10-Ca1d	High	100uM DFOM	1124.86	11.1262	H

Figure 5: Fluorescence lifetime (A) and free fractional component (B) of NADH in MCF10A vs. MCF10-Ca1d grown in low or high density collagen gels with 100uM DFOM. Charts on right indicate letter grouping via the Statistic software package SAS to more easily differentiate significantly different groups ($\alpha<0.05$).



Cell Type	Density	Treatment	Estimate	Standard Error	Letter Group
MCF10A	Low	control	1159.89	14.6899	BC
MCF10A	High	control	1148.61	14.6899	C
MCF10A	Low	10mM 2DG	1190.68	14.6899	A
MCF10A	High	10mM 2DG	1159.73	14.6899	BC
MCF10-Ca1d	Low	control	1154.18	14.6899	C
MCF10-Ca1d	High	control	1154.4	14.6899	C
MCF10-Ca1d	Low	10mM 2DG	1197.62	14.6899	A
MCF10-Ca1d	High	10mM 2DG	1179.19	14.6899	AB

Cell Type	Density	Treatment	Estimate	Standard Error	Letter Group
MCF10A	Low	control	65.3492	0.4485	AB
MCF10A	High	control	65.3794	0.4485	AB
MCF10A	Low	10mM 2DG	64.5351	0.4485	B
MCF10A	High	10mM 2DG	65.7202	0.4485	A
MCF10-Ca1d	Low	control	64.6251	0.4485	B
MCF10-Ca1d	High	control	64.9855	0.4485	AB
MCF10-Ca1d	Low	10mM 2DG	63.4064	0.4485	C
MCF10-Ca1d	High	10mM 2DG	64.5523	0.4485	B

Figure 6: Fluorescence lifetime (A) and free fractional component (B) of NADH in MCF10A vs. MCF10-Ca1d grown in low or high density collagen gels with 10mM 2DG. Charts on right indicate SAS letter grouping to more easily differentiate significantly different groups ($\alpha<0.05$).

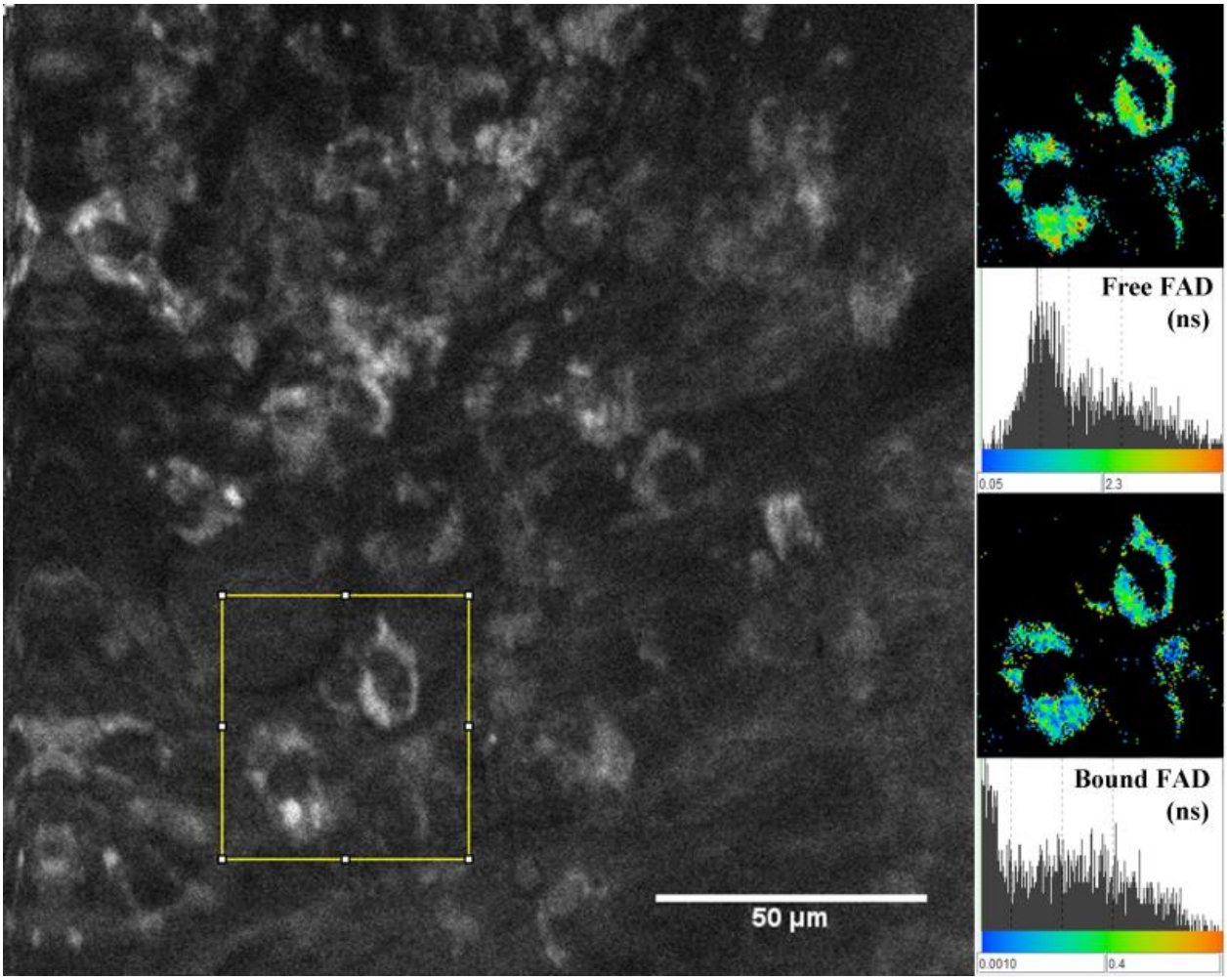


Figure 7: FLIM Measurement of FAD in a live mouse model. Fluorescence lifetime data from a female, PyVT positive, Col1a1 heterozygote mouse implanted with a mammary imaging window collected with a Nikon 40xW NA 1.15 objective at a zoom of 4 using 890nm excitation light and no emission filter.